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14. ABSTRACT
Mammalian hibernators display two unique characteristics that can be exploited for major advancements in surviving blood loss: (1) metabolism is carefully manipulated to achieve controlled reductions in oxygen demand of tissues for energy conservation, and the effect is fully reversible; and (2) hibernators tolerate massive changes in cardiac function, ventilation, tissue perfusion/ reperfusion, and intermediary metabolism that have similarities to shock and other trauma states, yet they are fully protected. Our team-oriented project integrated physiology, pathophysiology, and functional proteomics and metabolomics to identify novel candidate protection strategies based on these characteristics. We demonstrated that

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Hannah Carey
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Report Title

Translating the Hibernation Phenotype to Human Trauma Care

ABSTRACT

Mammalian hibernators display two unique characteristics that can be exploited for major advancements in surviving blood loss: (1) metabolism is carefully manipulated to achieve controlled reductions in oxygen demand of tissues for energy conservation, and the effect is fully reversible; and (2) hibernators tolerate massive changes in cardiac function, ventilation, tissue perfusion/ reperfusion, and intermediary metabolism that have similarities to shock and other trauma states, yet they are fully protected. Our team-oriented project integrated physiology, pathophysiology, and functional proteomics and metabolomics to identify novel candidate protection strategies based on these characteristics. We demonstrated that hibernators are highly protected against severe blood loss and mesenteric ischemia-reperfusio injury, and we identified several candidate proteins and metabolites that may contribute to hibernation-induced protection in these models.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Kurtz, C.C., S.L. Lindell, M.J. Mangino and H.V. Carey. 2006. Hibernation confers resistance to intestinal ischemia-reperfusion injury. Amer. J. Physiol. Gastrointest. Liver Physiol. 291:G895-901.

Serkova, N.J., J.C. Rose, L. E. Epperson, H.V. Carey and S.L. Martin. 2007. Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. Physiological Genomics 31:15-24.

Martin, S.L, L.E. Epperson, J.C. Rose, C.C. Kurtz, C. Ane and H.V. Carey. 2008. Proteomic analysis of the winter-protected phenotype of hibernating ground squirrel intestine. Amer. J. Physiol. Regul. Integr. Comp. Physiol. 295: R316-R328

Number of Papers published in peer-reviewed journals: 3.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Fleck, C.C., Lindell, S.L., Luterbach, K.J., Mangino, M.J. and H.V. Carey. 2004. Hibernation confers resistance to intestinal ischemia/reperfusion injury. Poster presentation at Life in the Cold: Evolution, Mechanisms, Adaptation and Application. Twelfth International Hibernation Symposium, Seward, AK

Fleck, C.C and H.V. Carey. 2005. Hibernation alters mucosal cytokine expression in ground squirrel intestine. Experimental Biology 2005 Abstract #406.13. [accessed at http://select.biosis.org/faseb/eb2005_data/FASEB001431.html].

Fleck, C.C and H.V. Carey. 2005. Hibernation confers resistance to organ damage following intestinal ischemia-reperfusion. Gastroenterology 128:A119.

Carey, H.V., K.T. Potter, T.L. Peters, L.E. Epperson, J.C. Rose and S.L. Martin. 2006. Hibernating mammals have enhanced survival and reduced gut damage after hemorrhage. The FASEB J, 20, Abstract # 903.1.

Nelson, C.J. and H.V. Carey. 2007. Metabolomic analysis of plasma from hibernating and active ground squirrels. Experimental Biology 2007 Meeting, Abstract #929.5. FASEB J. 21:965.14

Hengen, K.B., S.M. Johnson, H.V. Carey and M. Behan. 2007. Neural control of cardiorespiratory function in ground squirrels during hibernation. Experimental Biology 2007 Meeting, Abstract #929.5. FASEB J. 21:965.15

Otis, J. P. and H.V. Carey. 2007. Hibernation induces expression of PPAR? in ground squirrel intestine. Experimental Biology 2007 Meeting, Abstract #929.5. FASEB J. 21:929.5

Number of Presentations: 7.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Nelson, C.N., Otis, J.P., Martin, S.L. and H.V. Carey. Analysis of the hibernation cycle using LC-MS based metabolomics in ground squirrel liver. In review, Physiological Genomics.

Number of Manuscripts: 1.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Clark J. Nelson	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Hannah V. Carey	0.30	No
Martin Mangino	0.20	No
James Southard	0.50	No
Robert Haworth	0.30	No
Jose Torrealba	0.10	No
FTE Equivalent:	**1.40**	
Total Number:	**5**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED	
Tonia Peters	1.00	No
Susanne Lindell	1.00	No
Kathy Potter	0.80	No
Michael Grahn	0.50	No
FTE Equivalent:	**3.30**	
Total Number:	**4**	

Sub Contractors (DD882)

1 a. Sandra L. Martin

1 b. Department of Cellular and Developmen
University of Colorado School of Medic
Aurora CO 80045

Sub Contractor Numbers (c):

Patent Clause Number (d-1):

Patent Date (d-2):

Work Description (e): Identify hibernation-specific protein expression using proteomic analysis.

Sub Contract Award Date (f-1):

Sub Contract Est Completion Date(f-2):

Inventions (DD882)

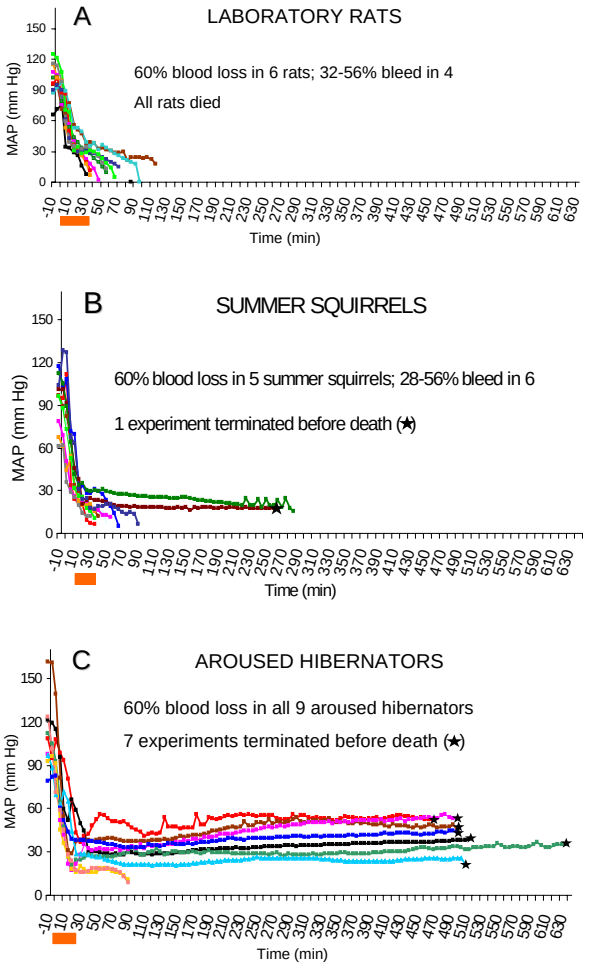
Translating the Hibernation Phenotype to Human Trauma Care
Award #W911NF-06-1-0106
H.V. Carey and S.L. Martin
September 24, 2008 Final Report

This report highlights the major accomplishments attained towards fulfilling the objectives of this project during the funding period of the award and through a no-cost extension period that ended April 30, 2007. We provided many details of data collected in our regular progress reports submitted during the funding period, and thus only highlights and conclusions are presented here.

Wisconsin Team – Carey Lab: In vivo shock model, gut ischemia model and metabolomics

1. Shock Model in Ground Squirrels: In keeping with the overall goal of the Surviving Blood Loss Program, we demonstrated that aroused hibernators live much longer after severe hypovolemic challenge compared with rats or summer squirrels (Fig. 1), suggesting that there is a mechanism that enhances survival under trauma conditions that is associated with the hibernation phenotype. We used Sprague-Dawley rats, spring/summer squirrels (late March – early June) and hibernating squirrels during an interbout arousal (T_b ~ 37°C). Body masses were 100–200 g. Animals were anesthetized with isofluorane, and after placement of catheters (aortic and vena cava for measurement of mean arterial pressure (MAP) and for blood withdrawal, respectively) external heating was removed. Blood was withdrawn (goal was 60% of total blood volume) over a 30-40 min period and MAP, heart rate (HR) and respiratory rate (RR) were recorded. No replacement fluids were provided. 60% blood removal was achieved in all hibernators, but some rats and summer squirrels died before this amount could be removed (see Figures for details). Endpoint was death (respiratory arrest and irreversible fall in MAP).

Fig. 1: Mean arterial pressure during and after hypovolemic challenge in rats (A), summer squirrels (B) and aroused hibernators (C). Hemorrhage time indicated by red bar on x axis. Some experiments were terminated by investigators if an animal survived for at least 4 h post bleed (indicated by star). These animals were considered long survivors. X axis scaled to show differences among groups.



All rats and most summer squirrels (9 of 11) did not survive 60% blood loss longer than ~1h post hemorrhage. In those animals MAP fell after hemorrhage and continued to decline rapidly until death. Two of the 9 hibernators died shortly after the end of hemorrhage, but the remainder lived for at least 8h; maximal survival times are unknown because those experiments were terminated prior to death. For hibernators that survived >8h, MAP fell during hemorrhage but was then maintained or increased slightly for the remainder of the experiment. T_bs of all animals gradually fell during hemorrhage. T_b remained ~24°C (room temperature) for many hours in hibernators that survived >8h. For rats and most summer squirrels, T_bs never reached the lower T_bs of the hibernators, due to insufficient time for cooling before death. Note that the hibernators had been without food or water for at least several weeks before they were used in experiments (hibernating squirrels normally do not eat or drink during the hibernation season), whereas the rats and summer squirrels were fully hydrated until

use and had eaten the day before. These preliminary results suggest that after severe hypovolemic challenge, aroused hibernators activate mechanisms that maintain MAP at a relatively high level and do so for long periods of time.

Compared with the consistent response of rats to this hypovolemic challenge, ground squirrels displayed variability in their resistance to 60% blood loss. In a separate group of squirrels studied in late summer/early fall, the response to 60% blood loss (not shown) was more similar to that in aroused hibernators than to the spring/early summer squirrels. Thus, hibernation-induced protection is not dependent on recent episodes of deep torpor, but appears to be gradually induced as squirrels prepare for the upcoming hibernation season. This makes the transition of protective mechanisms gleaned from hibernation to the clinical setting more feasible for humans, since profound hypothermia is not required.

2. Gut Ischemia-Reperfusion Model in Ground Squirrels: One organ system that is thought to contribute to multi-organ failure in response to massive blood loss in the gut, and it is possible that enhanced survival of hibernators in the shock model is due, at least in part, to minimizing massive cytokine release and subsequent multiple organ failure. In our project we indeed demonstrated that the hibernation phenotype provides a high degree of protection against intestinal ischemia-reperfusion injury compared with summer squirrels or rats, and this work has been was published in 2006 (Kurtz et al., 2006). Our collaborator Sandy Martin and her team in Colorado followed up this work by conducting proteomic analysis on tissues collected from summer and hibernating squirrels subject to intestinal I/R. That study, published in 2008 (Martin et al., 2008) identified several proteins that increased in expression levels in hibernator gut that may contribute to this protection. The manuscript is submitted with this report, but in brief, potential candidate proteins for hibernation-induced protection include albumin (free radical scavenger), apolipoprotein A-1 (anti-inflammatory and anti-atherogenic), heat shock protein 70 (chaperone/stress resistance) and HMGCS2 (may enhance energy status in gut via production of ketone bodies).

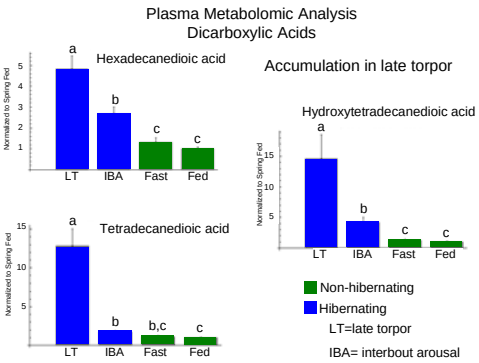
The Wisconsin team also followed up the Kurtz et al. 2006 study by testing the role of the transcription factor peroxisome proliferator-activated receptor- γ (PPAR-γ). PPARs, including PPAR-γ are activated by fatty acid and fatty acid metabolites. Activation of PPAR-γ has been demonstrated in other animal models to be protective against I/R injury as well as hemorrhagic shock. Because certain fatty acids and their metabolites known to be PPAR agonists are elevated during the hibernation season (as revealed by our metabolomic analyses and other studies), we hypothesized that hibernators use these molecules not only for their energetic benefits but also as signaling molecules that can provide protection against endogenous, and exogenous stress states. Using agonists and antagonists of this signaling pathway in hibernating and summer squirrels subject to an intestinal loop I/R model, we were unable to demonstrate a significant effect of PPAR-γ in mediating hibernation-induced protection. However, we still feel that bioactive lipids such as those that serve as endogenous ligands for PPARs may play a role in hibernation-induced protection from stress and trauma. It is very reasonable to suggest, given that hibernators switch to a “lipid-centric” metabolism in winter, that in addition to providing energy during the winter fast, lipid metabolites from the substantial adipose stores of hibernators are involved in critical signaling pathways that contribute to maintaining cellular, tissue and organ viability. Furthermore, our metabolomic studies have revealed significant changes in several lipid metabolites in liver and plasma during the seasonal hibernation cycle (see below).

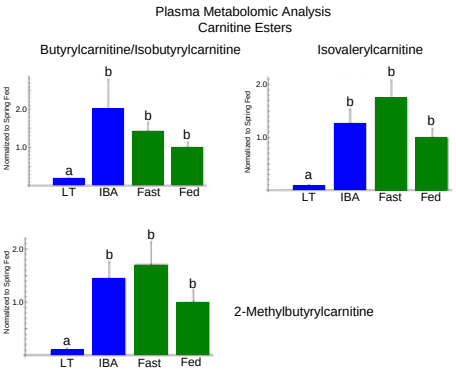
3. Seasonal upregulation of apolipoprotein A-I and HDL cholesterol and potential roles in hibernation-induced protection. Apo A-I, a protein produce by the liver and intestine and secreted into blood is the major lipoprotein associated with high density lipoprotein (HDL). The Martin lab, using 2-D DIGE proteomics, had identified Apo A-I as one of the proteins overexpressed in the gut of hibernating vs. non-hibernating squirrels subject to ischemia-reperfusion or sham surgery. We confirmed this with western blotting, and all of these results were published in early 2008 (Martin et. al., 2008). We subsequently found that Apo A-I is also upregulated in 13-lined ground squirrel liver and intestine (the two organs which produce it) and, importantly, in plasma where it can circulate to organs and tissues, and potentially be a key mediator that reduces inflammatory responses and organ dysfunction in trauma conditions. Apo A-I has been associated with protection in a variety of disease states. It is primarily responsible for the antiatherogenic property of HDL particles, has been shown to have

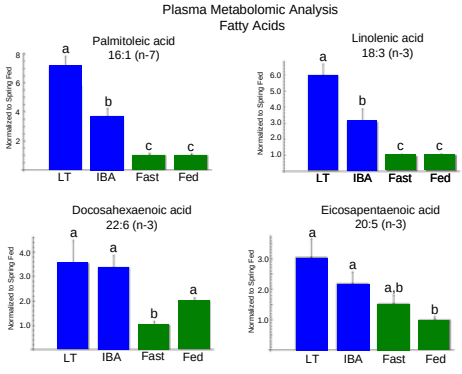
antioxidant and anti-inflammatory activities, and can bind bacterial lipopolysaccharide and inhibit its activity in vitro and in vivo. Furthermore, administration of HDL particles (Apo A-I in association with phospholipids) in a rat model of hemorrhage and resuscitation attenuated organ injury and dysfunction, and reduced inflammatory responses upon (Gillian et al. 2001). There is intense activity in the research community and industry to develop Apo A-I mimetics to enhance cardiovascular health. The discovery that this protein is naturally increased during hibernation in the two organs that produce it suggest that it plays an important role in cardiovascular protection during the hibernation season. Understanding how ground squirrels seasonally increase production of Apo A-I should facilitate development of therapeutic strategies to enhance its endogenous levels in humans at risk for vascular disease, shock and other pathologies. In studies begun near the end of the award period and currently ongoing in the laboratory we have discovered that levels of HDL cholesterol are increased in plasma of hibernating squirrels compared with summer, as would be expected given the elevation in Apo A-I production.

4. Mass spectrometry-based metabolomics: We used LC-MS based metabolomics to identify small molecules (metabolites) that characterize the hibernation state in liver, plasma and brain and thus reveal potential candidate molecules that contribute to hibernation-induced protection from early mortality after severe blood loss and other trauma sates, or may provide insight into mechanisms of inducing metabolic suppression in hibernation. A manuscript reporting results of liver metabolomic analysis in hibernating and active ground squirrels is in the review process at *Physiological Genomics* (Nelson et al.) and a copy is submitted with this report. A second manuscript reporting results of plasma metabolomic analysis in hibernating and active ground squirrels is in preparation and should be submitted for publication by November 1, 2008. Several promising plasma metabolites have now been identified and validated with standards (some shown below in figures); these are potential targets for inducing the hibernation state and/or the stress protection induced by hibernation. Substantial reduction of plasma short chain acyl carnitine esters are observed in torpor compared to aroused states whereas we see a striking accumulaton of dicarboxylic acids in late torpor (both of these patterns are consistent with results in liver, see Nelson et al. manuscript). Accumulation of dicarboxylic acids can result from incomplete fatty acid oxidation and high concentrations of these acids are toxic to the cell. A number of lipids, including omega-3 fatty acids, are elevated during hibernation and may exert protective effects (DHA, EPA). The elevation in another fatty acid, palmitoleic acid (C16:1n7) in hibernators is very interesting, as recent research has identified this monounsaturated fatty acid as a major lipid signaling hormone produced from adipose tissue that controls several metabolic activities in liver and muscle, and suppresses obesity-associated inflammation (Cao et al. *Cell* Sept 19, 2008). As dietary sources of palmitoleate are normally very low (as hibernators do not ingest food), elevations in palmitoleate are derived from *de novo* lipogenesis in adipose tissue.

We also carried out a metabolomic analysis of brain tissue in hibernators during the funding period, and analysis on that data set is continuing.







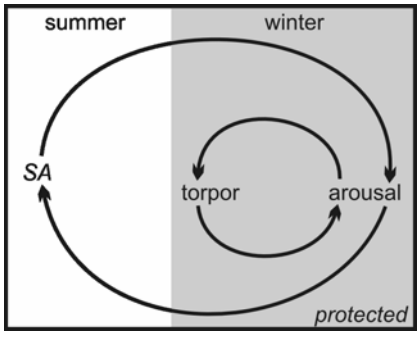
Colorado Team- Martin Lab Proteomics: Summary of highlights

- The hypothesis underlying our work was that changes in the proteome between summer and winter animals were responsible for the protected phenotype that is associated with hibernation in ground squirrels. Thus our goal was to use proteomics methodologies to identify proteins that differed between summer and winter in ground squirrels. Our milestone was to identify two such "hibernation-associated" proteins in the first year, which was easily achieved.

- We used two methods to quantitatively assess protein differences: 1) DIGE (fluorescent dye labeling followed by 2D gel electrophoresis), and 2) ^{15}N metabolic labeling, both using LC-MS/MS to identify the proteins. Numerous protein differences were found in several tissues examined. Our first report of these differences in gut tissue was published recently (Am J Physiol Regul Integr Comp Physiol 295: R316-28, 2008), and three additional manuscripts are in various stages of preparation to report the additional data obtained from liver and brain.

- The results of our NMR-based metabolomics study to compare abundant metabolites in the livers of summer active animals to those in late torpor and entrance phases of the torpor arousal cycle was published (Physiological Genomics, 31:15-24, 2007).

Data from both metabolomic and proteomic screens provided strong evidence for the shift of metabolic fuel from carbohydrate-based in summer to fatty acid-based in winter, and the elevation in winter of proteins that are typically associated with protection (eg heat shock proteins). There is also a strong signature of amino acid recycling and avoidance of urea generation in addition to several other factors whose roles are not as immediately clear. We are reluctant to make any sweeping conclusions about the key pathways involved in hibernation with the data uncovered in this study, because only the most abundant proteins and metabolites could be quantified with the methods used. Perhaps the most important outcome of this work for us was a deeper understanding of the cyclical nature of the physiological changes associated with hibernation and our realization that they could be modeled differently from our previous thinking, as depicted to the right. This new working model, the two switch model, now guides our work to reveal the molecular basis of hibernation. The first switch is between summer and winter mode, achieved by differential gene expression, which confers the protected phenotype of winter. The second switch, between torpor and arousal, is only enabled when the first is in winter position and is much less dependent on differential gene expression. We strongly believe that an effort to generate a complex microarray that can be used for deep probing of changes in the transcriptome across timepoints designed around this model offers the best prospect for uncovering the molecular pathways that are used to orchestrate the hibernating phenotype.



A two switch model for ground squirrel hibernation.

Summary of manuscripts resulting from this funding:

Kurtz, C.C., S.L. Lindell, M.J. Mangino and H.V. Carey. 2006. Hibernation confers resistance to intestinal ischemia-reperfusion injury. *Amer. J. Physiol. Gastrointest. Liver Physiol*. 291:G895-901.

Serkova, N.J., J.C. Rose, L. E. Epperson, H.V. Carey and S.L. Martin. 2007. Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. *Physiological Genomics* 31:15-24.

Martin, S.L, L.E. Epperson, J.C. Rose, C.C. Kurtz, C. Ane and H.V. Carey. 2008. Proteomic analysis of the winter-protected phenotype of hibernating ground squirrel intestine. *Amer. J. Physiol. Regul. Integr. Comp. Physiol.* 295: R316-R328

Nelson, C.N., Otis, J.P., Martin, S.L. and H.V. Carey. Analysis of the hibernation cycle using LC-MS based metabolomics in ground squirrel liver. In review, *Physiological Genomics*.

In addition, the Carey Lab has 2 papers in preparation and Martin Lab has an additional three for submission to peer-reviewed journals.